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A new member of α_1-adrenoceptor-coupled $G\alpha_h$ (transglutaminase II) family in pig heart: purification and characterization

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Abbreviations: G-protein, GTP-binding regulatory protein; PLC, phospholipase C; TGase, transglutaminase; GTPγS, guanosine 5'-O-(3-thiotriphosphate); CHAPS, (3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane-sulfonate; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; HEDG, 20 mM Hepes, 1 mM EGTA, 0.5 mM dithiothreitol, and 10% glycerol; HED, 20 mM Hepes, 1 mM EGTA, and 0.5 mM dithiothreitol; LDB, low detergent blotto; HDB, high detergent blotto

Abstract

We previously reported an identification of a 77-kDa GTP-binding protein that co-purified with the α_1-adrenoceptor following ternary complex formation. In the present paper, we report on the purification and characterization of this GTP-binding protein (termed $G\alpha_{h5}$) isolated from pig heart membranes. After solubilization of pig heart membranes with NaCl, $G\alpha_{h5}$ was purified by sequential chromatographies using DEAE-Cellulose, Q-Sepharose, and GTP-agarose columns. The protein displayed high-affinity GTPγS binding which is Mg^{2+}-dependent and saturable. The relative order of affinity of nucleotide binding by $G\alpha_{h5}$ was GTP> GDP > ITP >> ATP ≥ adenyl-5'-yl imidodiphosphate, which was similar to that observed for other heterotrimeric G-proteins involved in receptor signaling. Moreover, the $G\alpha_{h5}$ demonstrated transglutaminase (TGase) activity that was blocked either by EGTA or GTPγS. In support of these observations, the $G\alpha_{h5}$ was recognized by a specific antibody to $G\alpha_{h7}$ or TGase II, indicating a homology with $G\alpha_h$ (TGase II) family. These results demonstrate that 77-kDa $G\alpha_{h5}$ from pig heart is an α_1-adrenoceptor-coupled $G\alpha_h$ (TGase II) family which has species-specificity in molecular mass.

Keywords: α_1-adrenoceptor, G-protein, $G\alpha_h$, transglutaminase, heart

Introduction

α_1-Adrenoceptors, containing seven transmembrane domains typical in GTP-binding regulatory protein (G-protein)-coupled receptors, are found in most mammalian tissues. α_1-Adrenoceptors have several major physiological functions including smooth muscle contraction and changes of membrane currents in neurons and animal behavior (Minneman, 1988). Physiological responses of α_1-adrenoceptor are mediated by intracellular calcium mobilization through the activation of phosphoinositide-specific phospholipase C (PLC) via a toxin-insensitive G-protein (Wu *et al.*, 1992; Baek *et al.*, 1993; Das *et al.*, 1993). We have previously shown that α_{1B}-adrenoceptor stimulated a 69-kDa PLC through the activation of $G\alpha_h$ (transglutaminase II) (Baek *et al.*, 1993; Das *et al.*, 1993; Nakaoka *et al.,* 1994), indicating that $G\alpha_h$ is the signal mediator. In a recent report, Feng *et al.* (1996) demonstrated that the 69-kDa PLC was a proteolytic fragment of PLC-δ1 which was an effector of G_h signaling.

G_h, first purified from rat liver, appeared to be the 74-kDa GTP-binding protein, the α-subunit, associated with a 50-kDa protein (Im *et al.,* 1990). Recently, we have shown that the 78-kDa $G\alpha_{h7}$ purified from bovine heart was not only structurally and functionally similar to $G\alpha_h$ (Baek *et al.*, 1993) but also associated with a 50-kDa protein which is a modulator of GTP binding of $G\alpha_h$ (Baek *et al.*, 1993; Baek *et al.*, 1996a). These results suggested that $G\alpha_h$, but not an associated protein, may have species-specificity in molecular mass. In the previous paper, we have also reported an identification of a 77-kDa GTP-binding protein (termed $G\alpha_{h5}$) from pig liver and heart which co-purified with the α_1-arenoceptor following ternary complex formation (Yoo *et al.,* 1996). Recent studies on peptide sequence and immunological characterization have shown that $G\alpha_h$ family was a GTP-binding protein with transglutaminase II (TGase II) activity and receptor signaling function (Nakaoka *et al.,* 1994). In the present report, we describe that a new high molecular mass 77-kDa GTP-binding protein was purified from pig heart and biochemical and structural properties of purified protein was examined.

Materials and Methods

Chemicals

Protease inhibitors, (3-[(3-Cholamidopropyl)-dimethylammonio]-1-propane-sulfonate (CHAPS), heparin-agarose, dithiothreitol, N-acetylglucosamine, and Nonidet P-40 (NP-40) were obtained from Sigma. Wheat germ agglutinin-agarose and column chromatographic resins were from Pharmacia LKB Biotechnology Inc. Protein A-agarose, guanine nucleotides, and other nucleotides were obtained from Boehringer Mannheim. [α-^{32}P]GTP (3000 Ci/mmol), [^{3}H]putrescine (44.4 Ci/mmol), [^{3}H]prazosin (76 Ci/ mmol), and [^{35}S]GTPγS (1300 Ci/mmol) were from DuPont NEN. Other chemicals and biochemical materials were commercial preparations of the highest purity available.

Membrane preparations

Pig hearts were obtained from slaughterhouse (Doksan-Dong, Keumchun-Gu), and membranes were prepared by the method of Baek *et al.* (1993). All procedures were carried out at 0-4˚C. Homogenates of pig heart were prepared mechanically (Ultra-Turrax, Janke & Kunkel) at a ratio of 1:10 (w/v) in 10 mM Hepes buffer, pH 7.5, containing 250 mM sucrose, 5 mM EGTA, and protease inhibitors (bacitracin, 2 μg/ml; benzamidine, 100 μg/ml; leupeptin, 2 μg/ml; pepstatin A, 2 μg/ml; trypsin inhibitor, 2 μg/ml; phenylmethylsulfonyl fluoride, 2 μg/ml; and antipain, 20 μg/ml). The homogenates were filtered through four layers of cheesecloth and centrifuged at 500 *g* for 5 min. The supernatants were collected and centrifuged at 40,000 *g* for 1 h. The pellets were rehomogenized and recentrifuged three times with 50 mM Hepes buffer, pH 7.5, containing 10 mM $MgCl_2$, 5 mM EGTA, and the protease inhibitors listed above, and resuspended as 10 mg protein/ml in HEDG buffer (20 mM Hepes, 1 mM EGTA, 0.5 mM dithiothreitol, 10% glycerol, pH 7.5) containing 100 mM NaCl and protease inhibitors. The membrane suspensions were stored at -80˚C until use.

Purification of 77-kDa GTP-binding protein $G\alpha_{h5}$ from pig heart

The 77-kDa GTP-binding protein was purified by sequential column chromatographies using DEAE-Cellulose, Q-Sepharose, and GTP-agarose at 4˚C, and protease inhibitors (2 μg/ml each of bacitracin, leupeptin, pepstatin A, trypsin inhibitor, and phenylmethylsulfonyl fluoride, and 20 μg/ml each of benzamidine and antipain) were included in all buffers. During the purification, G-proteins were monitored by photoaffinity labeling with [α-^{32}P]GTP and [^{35}S]GTPγS binding. Glycerol (10%, v/v) was used to stabilize proteins. The purification protocol described in Table I is a representative of several independent experiments.

Pig heart membranes were washed once with HED buffer (20 mM Hepes, 1 mM EDTA, and 1 mM dithiothreitol, pH 7.5) containing 100 mM NaCl. After centrifugation at 40,000 *g* for 40 min, the membrane pellets were resuspended as 5 mg/ml protein in the same buffer, and solubilized in 250 mM NaCl at 4˚C for 1 h. The extract obtained by centrifugation at 40,000 *g* for 1 h was then diluted 4-fold with HED buffer containing 10% glycerol. The diluted extract was applied onto a DEAE-Cellulose column (200 ml) which had been equilibrated with HED buffer containing 0.05% CHAPS, 10% glycerol, and 100 mM NaCl. The column was washed with 500 ml of the column equilibrium buffer, and the retained material was eluted with HED buffer containing 0.05% CHAPS, 10% glycerol, and 400 mM NaCl. The pooled fractions from the DEAE-Cellulose column were applied onto the first Q-Sepharose column (5 × 10 cm) which had been equilibrated with HED buffer containing 70 mM NaCl. The column was washed with 200 ml of the equilibration buffer, the retained materials were eluted using 100 ml of a linear salt gradient (50-700 mM) in the same buffer, and 2 ml fractions were collected at a flow rate of 30-40 ml/h. The $G\alpha_{h5}$-containing fractions were pooled. The pooled fractions were diluted with HED buffer and the sample was loaded onto a Q- Sepharose column (10 ml). The column was washed with 50 ml of HED buffer containing 250 mM NaCl, and was eluted with 50 ml of a linear sodium chloride gradient (200-700 mM). Fractions (1.5 ml) were collected at a flow rate of 30 ml/h., the collected fractions diluted with HED buffer at a protein concentration of about 0.3 mg/ml, and the sample was loaded (6 ml/h) onto a GTP-agarose column (3.0 ml bed volume) which had been equilibrated with HED buffer. Runthrough fractions were collected and reloaded (6 ml/h). After the second loading, the column was washed (24 ml/h) with 20 bed volumes of the same buffer and $G\alpha_{h5}$ was eluted (24 ml/h) from the GTP column with 1 M KCl in HED buffer. The first five fractions were pooled and stored at -80˚C.

Binding assays and photoaffinity labeling

These assays were performed essentially as described previously (Baek *et al.*, 1993; 1996a; 1996b). The amount of α_1-adrenoceptor was determined using [^{3}H]prazosin binding after incubation at 30˚C for 30 min. Transglutami-nase activity was performed in the presence and absence of 100 μM $CaCl_2$ and 5 μM GTPγS by incorporation of [^{3}H]putrescine (0.1 mM) into N,N'-dimethylated casein (1%) at 20˚C for 30 min. For [^{35}S]GTPγS binding, samples were incubated with 1 μM GTPγS plus 1 μCi [^{35}S]GTPγS for 30 min at 30˚C. The photoaffinity labeling was performed in the presence of 5-10 μCi [α-^{32}P]GTP and 2 mM $MgCl_2$ in ice bath under 254 nm UV irradiation for 5-10 min.

Table I. Purification of 77-kDa GTP-binding protein $G\alpha_{h5}$ from pig heart. The values are a representative of several independent experiments.

Steps	Volume	Protein	[^{35}S]GTPγS bound	Specific activity	Recovery
	ml	mg	nmol	pmol/mg	%
Membranes	250	1,912.0	713.0	372.9	100.0
Membrane extract	200	273.9	137.7	502.7	19.3
DEAE-Cellulose	150	67.5	47.3	700.7	6.63
1st Q-Sepharose	100	11.4	12.7	1114.0	1.78
2nd Q-Sepharose	57	1.7	8.4	4941.2	1.18
GTP-Agarose	6	0.07	1.33	19000.0	0.19

Antibody experiments

Immunological cross-reaction experiments were performed essentially as described previously (Baek *et al.*, 1993; 1996b). Immunoblots using 1:500 diluted antibody were detected by Enhanced Chemiluminescence (Amersham, UK). Briefly, proteins were separated on 7.5-12% gels by SDS-PAGE, and then transferred to Immobilon-P (Millipore). The membranes were blocked for 1 h with LDB (low detergent blotto, 80 mM NaCl, 2 mM $CaCl_2$, 0.02% NaN_3, 0.2% NP-40, 50 mM Tris/HCl, pH 8.0, containing 5% nonfat dry milk) at room temperature, and were then incubated with LDB containing anti-TGase II antibody (1:500 dilution) for 1 h at room temperature. After washing three times with LDB, the membranes were incubated with anti-rabbit immunoglobulin, peroxidase-linked species-specific antibody (1:1000 dilution) in HDB (high detergent blotto, 2% NP-40 in LDB), for 1 h at room temperature. After washing three times, the membranes were subjected to procedures for Enhanced Chemilumine-scence (Amersham, UK). Protein A-agarose (binding capacity, 22 mg of rabbit IgG/ml of agarose) was used for immunoprecipitation.

Protein determination

Protein concentration was measured by the method of Bradford (1976) using ready-made Bio-Rad protein determination kit, using bovine serum albumin as a standard.

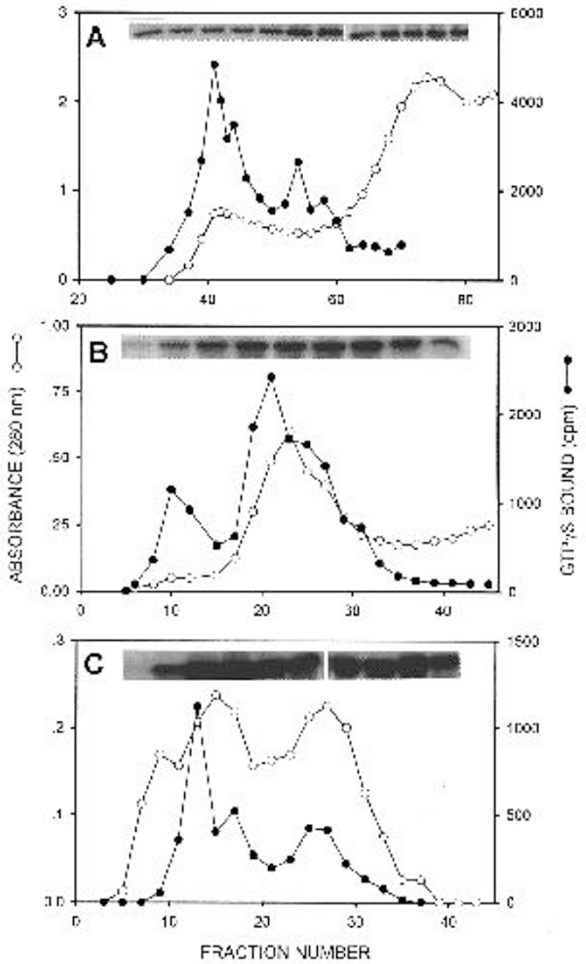

Figure 1. Elution profile of protein and GTPγS binding activity. An aliquot (50 μl) of each of the fractions was photolabeled with [α-^{32}P]GTP, and then analyzed by SDS-PAGE (10% gel) and autoradiography. *Panel A,* DEAE-Cellulose chromatography. The retained material was eluted with 400 mM NaCl. *Panel B,* the first Q-Sepharose chromatofraphy. The 77-kDa GTP-binding protein from the column was eluted in the range of 350-400 mM NaCl. The 40-kDa class of GTP- binding proteins were eluted at < 200 mM NaCl, as evaluated by the results of photolabeling with [α-^{32}P]GTP. *Panel C,* The Second Q-Sepharose chromatography. The 77-kDa GTP-binding protein was eluted in a range of 250-350 mM. *Insets* show autoradiogram of the 77-kDa GTP-binding protein in the major GTPγS binding peak, respectively.

Results

Purification of the 77-kDa GTP-binding protein $G\alpha_{h5}$

Purification of 77-kDa GTP-binding protein was made possible by the ability to photochemically modify G-proteins with [α-^{32}P]GTP, and then assess its molecular mass by SDS-PAGE and autoradiography. In addition, G-proteins were tracked throughout the purification procedures by their ability to irreversibly bind GTPγS

using [^{35}S]GTPγS as a tracer. For selective solubilization of the 77-kDa GTP-binding protein, salt concentration was critical for increasing specific solubilization (Baek *et al.*, 1993). The optimal salt concentration was 200-400 mM in HED buffer. An increase in the salt concentration (400-2000 mM) increased the GTPγS binding, however, the specific binding activity decreased due to increase of total protein extracted from the membranes. It should be noted that the 77-kDa GTP-binding protein could be solubilized with salt alone, but the protein became easily aggregated in the absence of the detergent, CHAPS, and lost the ligand binding activity within less than 1 week.

The sequential chromatographic steps used to purify $G\alpha_{h5}$ from pig heart is shown in Figure 1. A fixed concentration of NaCl (400 mM) was applied to elute the bound material from DEAE-Cellulose (Figure 1A). Ion-exchange chromatography allowed the G-protein to be concentrated. The elution profile of the 77-kDa GTP-binding protein was similar to that of $G\alpha_{h7}$ purified from bovine heart membranes with Q-Sepharose (Figure 1B). The washing of the second Q-Sepharose column with high salt (250 mM) resulted in separation of most protein from the 77-kDa GTP-binding protein (Figure 1C). Further purification and concentration of the 77-kDa GTP-binding protein were achieved using GTP-agarose affinity chromatography. The overall results on the purification scheme are sum-marized in Table I.

A 50-kDa protein which did not bind GTP consistently co-eluted with the 77-kDa GTP-binding protein, except on GTP-agrose column (Data not shown). The efficiency of photoincorporation of [α-^{32}P]GTP into the $G\alpha_{h5}$ was <2% which is somewhat lower than that observed by other investigators (Sternweis *et al.*, 1981; Linse and Mandelkow, 1988). However, GTPγS binding by $G\alpha_{h5}$ was linear with increasing protein concentrations (Data not shown). Photoaffinity labeling was dependent on UV irradiation time and temperature, and the purified protein was stable in the buffer for less than 3 weeks without a G-protein stabilizer. However, when aluminum fluoride was included, the protein was stable for a month, as mentioned earlier (Baek *et al.*, 1993).

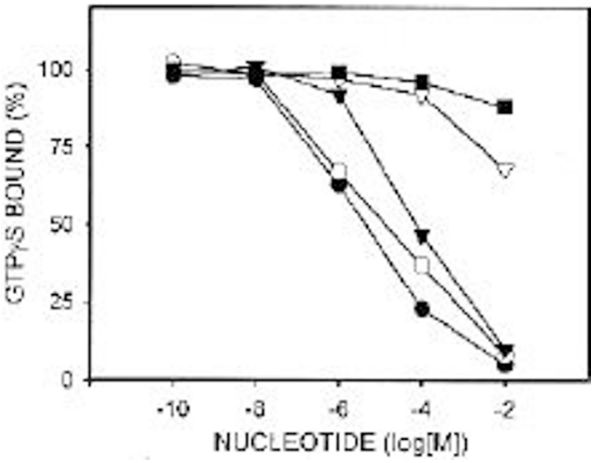

Figure 2. Competition by nucleotides for GTPγS binding by purified $G\alpha_{h5}$. Purified $G\alpha_{h5}$ (20 ng) was incubated in HED buffer containing 100 mM NaCl, 10 mM $MgCl_2$, 1 μCi of [^{35}S]GTPγS and nucleotides at 30˚C for 10 min. Datum shown is a representative of three independent experiments, and each point is an average of duplicate determinations. ●, GTP; ○, GDP; ▼, ITP; ▽, ATP; ■, App(NH)p.

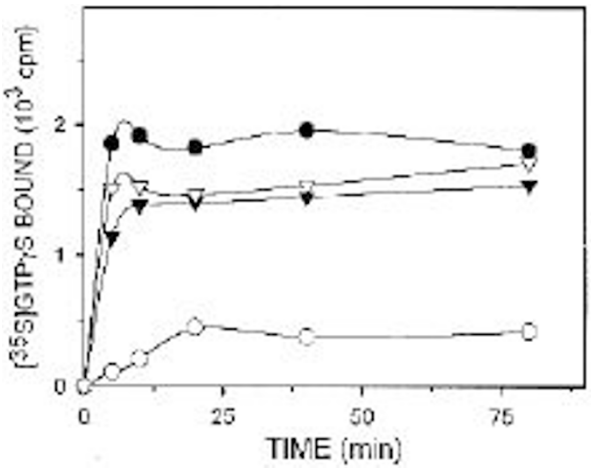

Figure 3. Mg^{2+} dependency of GTPγS binding by purified $G\alpha_{h5}$. Purified $G\alpha_{h5}$ (20 ng) was incubated with various concentrations of Mg^{2+} in HED buffer containing 100 mM NaCl and 1 μM [^{35}S]GTPγS at 30˚C for 10 min. Datum shown is a representative of three independent experiments, and each point is an average of duplicate determinations. $MgCl_2$ concentration: ○, 0 mM; ▼, 5 mM; ▽, 10 mM; ●, 20 mM.

Biochemical properties of 77-kDa GTP-binding protein $G\alpha_{h5}$

As shown previously (Yoo *et al.,* 1996), the photoaffinity labeling of the 77-kDa protein was competitive with GTP but not by ATP (Data not shown), indicating that the $G\alpha_{h5}$ has a specific binding site for guanine nucleotides. The specificity of the binding site was further determined using various nucleotides (Figure 2). The order of inhibition of nucleotides for the GTPγS binding by $G\alpha_{h5}$ was GTP > GDP > ITP >> ATP ≥ adenyl-5'-yl imidodiphosphate (App(NH)p). At 1 μM GTPγS (half-maximal binding: 0.25-0.30 μM under this condition), the half-maximal inhibition of GTPγS binding by nucleotides was GTP, ~20 μM; GDP, ~50 μM; and ITP, ~100 μM. These results indicate that $G\alpha_{h5}$ is a specific GTP-binding protein. Binding of guanine nucleotides by other G-proteins is Mg^{2+}-dependent. In the presence of Mg^{2+}, binding of nonhydrolyzable GTP analogues, such as GTPγS, is virtually irreversible (Gilman, 1987). Mg^{2+} dependence of GTPγS binding by $G\alpha_{h5}$ is shown in Figure 3, and the result indicates that the extent of binding increases with increasing Mg^{2+} concentration over the range of 0-20 mM. The purified

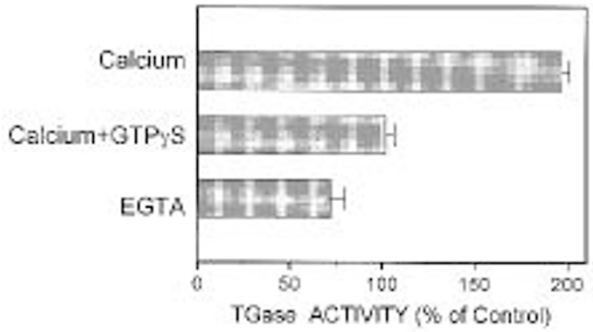

Figure 4. Transglutaminase activity of purified $G\alpha_{h5}$. Purified $G\alpha_{h5}$ (20 ng) was incubated with 2 mM EGTA, 100 μM calcium, or 100 μM calcium plus 5 μM GTPγS, and then TGase activity was measured. The TGase activity is expressed as a percentage of purified $G\alpha_{h5}$ without ligands (control). The data shown are means ± S.D of three independent experiments each in triplicates.

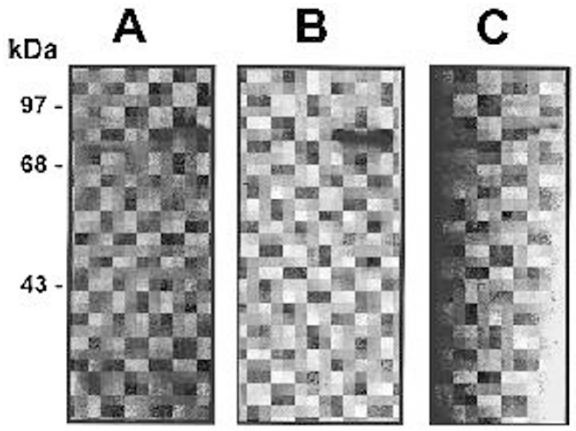

Figure 5. Determination of immuno-reactivity of purified $G\alpha_{h5}$. *Panel A*, Silver staining of purified guinea pig liver TGase II and purified protein from pig heart. *Panel B*, immunoprecipitation by anti-$G\alpha_{h7}$ antibody using Protein A-agarose. Purified protein was applied to a dried G-25 column to remove salt. Prior to performing the immunoprecipitation the samples were photolabeled with 10 μCi of [α-^{32}P]GTP in the presence of 2 mM $MgCl_2$ for 5 min by UV irradiation. *Panel C*, immunoblot by TGase II antibody. *left lane,* purified guinea pig liver TGase II; *right lane,* purified protein from pig heart.

$G\alpha_{h5}$ protein contained TGase activity which was blocked by either EGTA or GTPγS (Figure 4).

Immunological cross-reactivity of purified 77-kDa GTP-binding protein $G\alpha_{h5}$

As mentioned above, functional properties of the pig heart 77-kDa GTP-binding protein $G\alpha_{h5}$ are similar to the $G\alpha_h$ family. The distinct difference is the molecular mass (Figure 5A). To evaluate whether pig heart $G\alpha_{h5}$ is distinct from the $G\alpha_h$ family, anti-$G\alpha_{h7}$ antibody and TGase II antibody were used to test an immunological cross-reactivity. As demonstrated in Figure 5B and 5C, when the purified $G\alpha_{h5}$ from pig heart and TGase II from guinea pig were subjected to immunoprecipitation and immunoblotting, the pig heart $G\alpha_{h5}$ was effectively cross-reacted with both antibodies. Moreover, their molecular masses also showed distinct difference. Pig heart $G\alpha_h$ has higher molecular mass than TGase II from guinea pig. These results clearly indicated that the 77-kDa GTP-binding protein $G\alpha_{h5}$ in pig heart is homologous to other $G\alpha_h$ (TGase II) family, and $G\alpha_h$ family has species-specific molecular mass.

Discussion

Evidence for an involvement of a G-protein in α_1-adrenoceptor signaling was first presented by Goodhardt *et al.* (1982). These investigators demonstrated that α_1-adrenergic agonist binding in rat liver membrane was modulated by guanine nucleotides. Subsequently, Uhing *et al.* (1986) and others (Boyer *et al.*, 1984 ; Lynch *et al.*, 1985) provided additional evidence and further demonstrated that pertussis toxin did not alter phosphatidylinositol turnover resulting from α_1-agonist stimulation of phospho-lipase C in the presence of guanine nucleotides.

We have previously shown that $G\alpha_h$ family (TGase II) not only couples to α_1-adrenoceptor and oxytocin receptor (Baek *et al.*, 1993; 1996b), but also modulates a large conductance Ca^{2+}- activated K^+ channel and PLC (Das *et al.,* 1993; Feng *et al.*, 1996; Lee *et al.,* 1997; Park *et al*, 1998). In the present paper, the purification of the 77-kDa GTP-binding protein $G\alpha_{h5}$ from pig heart membranes was carried out by applying a technique involving direct photoaffinity labeling of GTP-binding proteins with [α-^{32}P]GTP. The purified 77-kDa protein, like the α-subunits of other heterotrimeric G-proteins, has a binding site for guanine nucleotides and binds nucleotides in a Mg^{2+}-dependent manner, with an appropriate order of affinities *viz.* GTP > GDP > ITP >> ATP ≥ App(NH)p. These results indicate that the properties of $G\alpha_{h5}$ are similar to those of the 40-kDa class of heterotrimeric G-proteins (Northup *et al.*, 1980; Bokoch *et al.*, 1984; Sternweis and Robishaw, 1984). Furthermore, as shown previously (Nakaoka *et al.,* 1994), the purified protein demonstrated TGase activity which was blocked by either EGTA or GTPγS. The identity of $G\alpha_{h5}$ with TGase II was further indicated by the observation that antibodies to guinea pig liver TGase II or $G\alpha_{h7}$ recognized both purified pig heart $G\alpha_{h5}$ and guinea pig liver TGase II. However, the distinct difference lay in the molecular mass. This study showed an existence of a high molecular mass G-protein family which has a species-specific molecular mass. The reason for species-specific differences should be studied further. Nevertheless, changes in the DNA level, *i.e.*, by alternative splicing, may be a possibility, because the same species contains the identical molecular

mass G_h, regardless of the origin of tissue (Baek *et al.,* 1993). Thus, it may be due to differences in the primary structures or post-translational modifications which may account for different control of α_1-adrenergic system in various species. Comparative studies on this molecular heterogeneity might provide an insight into structure-function relationship of $G\alpha_h$ family.

Acknowledgements

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